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Regulated production of an influenza virus spliced mRNA mediated by virus-specific products

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The influenza virus NS_2 mRNA is generated through processing by cellular enzymes of a transcript (the NS_1 mRNA) of virion RNA segment 8. Production of this mRNA is altered in cells infected with a mutant of influenza A (fowl plague) virus. The proportion of segment 8 transcripts which accumulated in a spliced form was found to be considerably lower in mutant virus-infected cells than in cells infected with wild-type virus, and the amplification in production of NS_2 mRNA relative to that of the NS_1 mRNA, which normally occurs during infection with wild-type virus, was not observed with the mutant. The NS_1 mRNA specified by the mutant virus has unaltered splice recognition sites and was apparently processed normally during a mixed infection with a strain of virus which is wild-type for production of NS_2 mRNA. These results suggest that the production of NS_2 mRNA is regulated by virus-specific products; these products may act by increasing the efficiency of splicing of NS_1 mRNA.

Key words: influenza virus/spliced mRNA/regulation of RNA splicing

Introduction

The mRNAs encoding the influenza virus M_2 and NS_2 polypeptides are produced in infected cells through splicing of primary transcripts from virion RNA segments 7 and 8 respectively (Inglis *et al.*, 1980; Lamb and Lai, 1980; Inglis and Brown, 1981; Lamb *et al.*,1981). These primary transcripts, which are also functional mRNAs (encoding in the case of segment 7, matrix protein, and in the case of segment 8, the NS_1 protein), are almost certainly formed from their unspliced precursors through the action of cellular RNA processing enzymes, since the nucleotide sequences at the intron/exon boundaries of the precursor mRNAs (Lamb and Lai, 1980; Lamb *et al.*, 1981) resemble those found at the splice sites of higher eukaryotic mRNA precursors (Mount, 1982). In addition, most of these splicing signals are correctly recognised in precursor mRNAs that are expressed from cloned copies of vRNA segment 7 or 8, either in cells infected with recombinant SV40 viruses (Lamb and Lai, 1982, 1984), or in cells containing influenza virus-specific DNA which is stably integrated within cellular DNA (M. Inglis, personal communication). However, there is also evidence to suggest that the production of influenza virus spliced mRNAs can be enhanced by virus-specific products. During the replication of influenza virus there is an increase in the abundance of the virus-specific spliced mRNAs relative to that of their unspliced precursors, the increase being particularly obvious for the vRNA segment 8-specific spliced mRNA encoding the NS_2 polypeptide (Inglis and Brown, 1984). When cells are treated with inhibitors of protein synthesis throughout infection, the relative abundance of spliced mRNA remains low (Lamb *et al.*, 1978; Inglis, 1978; Inglis and Brown, 1984). One interpretation of these observations is that virus-specific products may somehow regulate the production of spliced mRNA.

We anticipated that if virus-specific products are indeed responsible for the regulated production of influenza virus spliced mRNAs, then it might be possible to identify viruses that encode altered versions of such products and consequently display a different pattern of spliced mRNA production during virus replication. We report here the identification and characterisation of a mutant of influenza A [fowl plague virus (FPV)] in which the regulated production of the NS_2 mRNA is defective.

Results

Accumulation of NS_2 mRNA in wild-type and mutant virus-infected cells

The accumulation of NS_2 mRNA and of its unspliced precursor, NS_1 mRNA, was studied in cells infected with either wild-type FPV (influenza A/FPV/Rostock/34) or with the virus tsC mN3, which is a temperature-sensitive mutant of the wild-type strain (Almond *et al.*, 1979). The cells chosen for this study were chick embryo fibroblast (CEF) cells which are a highly permissive system for growth of FPV (Breitenfeld and Schafer, 1957), and L cells which are abortive for FPV replication (Franklin and Breitenfeld, 1959), but in which the production of NS_2 mRNA is greatly enhanced during virus infection (Inglis and Brown, 1984). Cytoplasmic extracts were prepared from cells at intervals after infection, and the accumulation of the two vRNA segment 8-specific mRNAs was monitored by the technique of hybrid selection of reverse transcripts (Inglis *et al.*, 1980; Inglis and Brown, 1981). Polyadenylated mRNAs were isolated from the cell extracts and copied into radiolabelled DNA by reverse transcription using oligo(dT) as a primer. DNA copies of NS_1 mRNA and NS_2 mRNA were selected for gel analysis by hybridisation to immobilised plasmid DNA containing a cloned copy of the 3′-terminal 53 nucleotides of vRNA segment 8 (Smith, 1985). Only complete reverse transcripts of NS_1 mRNA and NS_2 mRNA were detected by this procedure (Figure 1) because incomplete reverse transcripts do not contain sequences that are homologous with the 3′ terminus of vRNA segment 8.

In wild-type virus-infected CEF and L cells the abundance of NS_2 mRNA, relative to that of NS_1 mRNA, increased during the course of infection (Figure 1), as reported previously (Inglis and Brown, 1984). In contrast, the relative abundance of NS_2 mRNA did not increase during mN3 infection of either cell type, remaining at a level similar to that observed in wild-type virus-infected cells at 2 h after infection. This alteration in the regulation of NS_2 mRNA production may account for a previous report in which no synthesis of the NS_2 protein could be detected in mN3-infected CEF (Wolstenholme *et al.*, 1980).

One explanation for the altered production of NS_2 mRNA in

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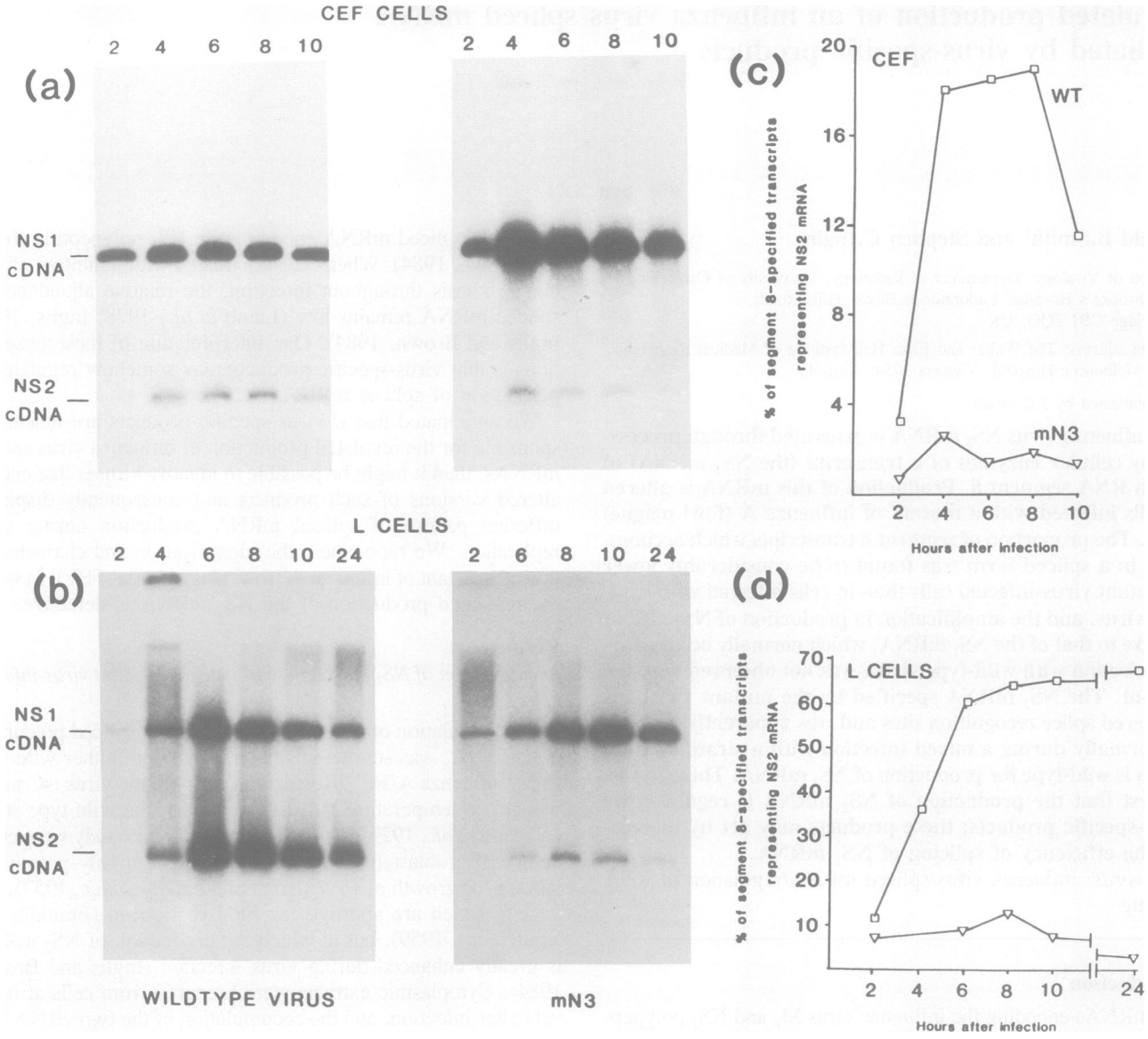

Fig. 1. Accumulation of vRNA segment 8-specific mRNAs in (**a**) CEF and (**b**) L cells infected with wild-type virus (left-hand panels) or mN3 (right-hand panels). Cells were infected at a multiplicity of 10 p.f.u./cell and harvested at the times indicated (h post-infection). Poly(A)-containing RNA was isolated from the cytoplasmic fraction and equivalent amounts were reverse-transcribed in the presence of [α-^{32}P]dCTP. The reaction products were hybridised with nitrocellulose filters bearing immobilised vRNA segment 8-specific DNA (pFPV 82A) and cDNA species which hybridised specifically were eluted, separated on a 7.5% polyacrylamide gel and detected by autoradiography. Tracks were scanned using a densitometer and the proportion of total segment 8-specific transcripts represented as NS_2 mRNA (NS_2/NS_2+NS_1) was calculated. The results are presented in panels (**c**) (CEF) and (**d**) (L cells).

mN3-infected cells is that mN3 encodes a defective version of a product which is normally responsible for the regulation of NS_2 mRNA production. Alternatively, the mN3 vRNA segment 8-specific mRNAs might contain mutations which alter their relative stability or reduce the suitability of NS_1 mRNA as a substrate for RNA splicing.

The nucleotide sequence of mN3 vRNA segment 8

To discriminate between these possibilities, we first deduced the nucleotide sequences of mN3 vRNA segment 8 and compared it with that of wild-type virus. Our aim was to discover if the NS_1 mRNA encoded by mN3 contains any mutations which might be expected to have a *cis*-acting effect on its processing by RNA splicing. DNA copies of mN3 and wild-type virus vRNA segment 8 were synthesised by reverse transcription, cloned into replicative form DNA of bacteriophage M13 mp8 and the nucleotide sequence of one mN3-derived and one wild-type virus-derived clone was determined by dideoxynucleotide sequencing (Materials and methods).

The deduced nucleotide sequence of the mutant virus vRNA segment 8 was identical to that of wild-type virus except for a single difference at nucleotide 150; this nucleotide was an adenosine residue in the wild-type virus sequence and a guanosine residue in the mN3 sequence (Figure 2a). Although it seemed unlikely that this difference was an artefact of the cloning procedure, since it was present in two independently derived clones of mN3 vRNA segment 8, this region was also sequenced directly by primer extension on purified vRNA segment 8 from both the mutant virus and wild-type virus. The same nucleotide sequence difference at position 150 was observed (Figure 2b).

A common feature of all those mutations which have been shown to affect the splicing of higher eukaryotic spliced mRNA

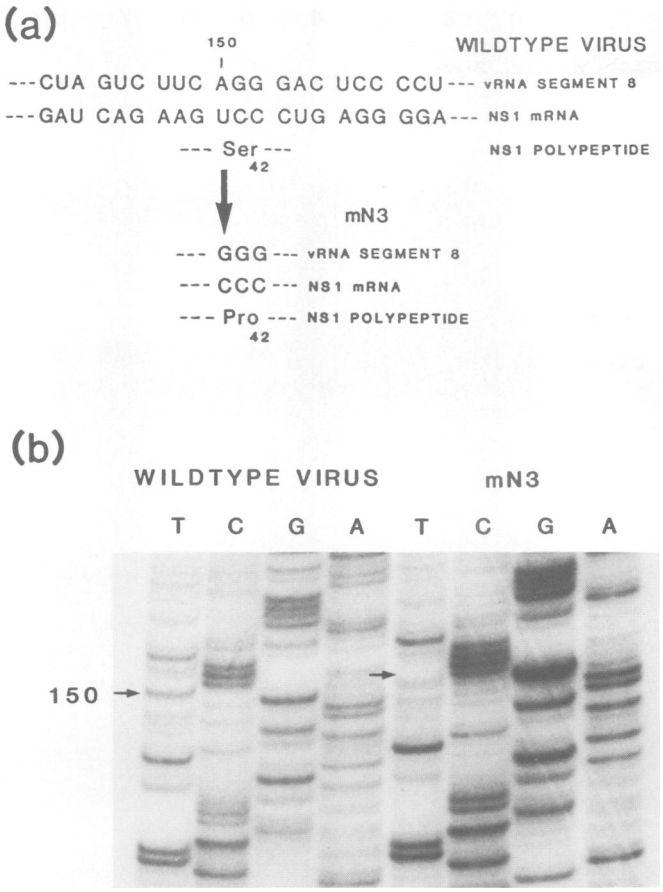

Fig. 2. Nucleotide sequence of wild-type virus and mN3 vRNA segment 8 in the region of nucleotide 150. (**a**) Representation of the single mutation which was detected in mN3 vRNA segment 8 (at nucleotide 150) and the predicted alteration to the structure of the mN3 NS_1 polypeptide (at residue 42). The nucleotide sequence of cloned DNA copies of wild-type virus and mN3 vRNA segment 8 was deduced by the dideoxynucleotide chain termination procedure (Materials and methods). (**b**) Direct sequencing of wild-type virus and mN3 vRNA segment 8 in the region of nucleotide 150. Purified vRNA segment 8 of wild-type virus or mN3 was reverse transcribed in the presence of a ^{32}P-labelled oligonucleotide complementary to the vRNA 3′ terminus, and each of the four dideoxynucleotides. Reaction products were separated on a 6% polyacrylamide buffer gradient gel and detected by autoradiography (Materials and methods). The products which terminate at nucleotide 150 are indicated.

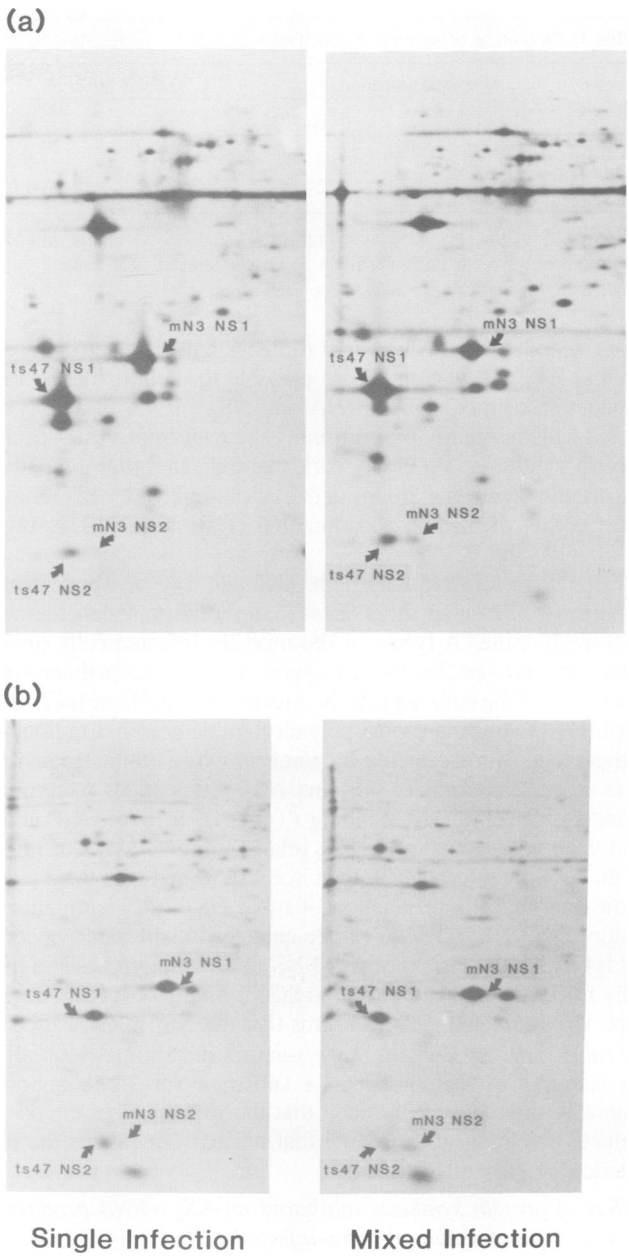

Fig. 3. Synthesis of vRNA segment 8-specific proteins in doubly infected cells. CEF (**a**) and L cells (**b**) were infected singly with mN3 or ts47, or with the two viruses together. In each case the total multiplicity of infection was 10 p.f.u./cell. Cells were incubated in medium containing [^{35}S]methionine (**a**) for 1 h from 3.5 h after infection or (**b**) for 30 min from 7 h after infection, and cell lysates were subjected to two-dimensional non-equilibrium pH gradient electrophoresis. Samples for electrophoresis were either pooled lysates containing equal quantities of the cells which were separately infected with mN3 or ts47 (single infection, left-hand panels), or a lysate of the cells which were infected with both mN3 and ts47 (mixed infection, right-hand panels). Radiolabelled proteins were detected by fluorography.

precursors is that they alter sequences within the precursor RNA which are related to the splice site consensus sequences (Mount, 1982) in such a way that they become either more closely or less closely related to the consensus (Goldsmith *et al.*, 1983; references in Wieringa *et al.*, 1984). The NS_1 mRNA encoded by mN3 must contain a mutation at nucleotide 150 (Figure 2a), but this mutation does not alter the consensus-related sequences at nucleotides 56/57 and 528/529 which direct the splicing of NS_1 mRNA into NS_2 mRNA; furthermore the mutation occurs within a region that is unrelated to either of the splice site consensus sequences. Hence, there is no reason to suspect that this mutation has a *cis*-acting effect on the splicing of the mutant virus NS_1 mRNA.

Production of mN3 NS_2 mRNA in doubly infected cells

If the reason for the deficiency in NS_2 mRNA production, relative to that of NS_1 mRNA, in mN3-infected cells is that the mutant virus encodes a defective version of a product which normally promotes the production of NS_2 mRNA, then during a mixed infection with a virus that encodes a wild-type version of this product, complementation should restore mN3 NS_2 mRNA production to wild-type levels. If, on the other hand, the deficiency is a *cis*-acting effect of the mutation at nucleotide 150 of mN3 NS_1 mRNA, then the relative production of mN3 NS_2 mRNA should still be deficient in doubly infected cells. To carry out this experiment we took advantage of the fact that another mutant of FPV, tsC 47 (Almond *et al.*, 1979), which is wild-type for spliced mRNA production at the permissive termperature, encodes NS_1 and NS_2 proteins that can be distinguished from

Table I. Proportion of segment 8-specified polypeptides represented by NS_2[a]

Infected cell type	Single infection		Mixed infection	
	nM3	ts47	mN3	ts47
CEF	0.8%	8.2%	7.3%	9.5%
L	3%	27%	12%	17%

[a]The density of the spots corresponding to NS_1 and NS_2 in Figure 3a and b was measured using a Beckman Du-8 spectrophotometer. The ratio NS_2/NS_2+NS_1 was then calculated for each virus.

those encoded by mN3 by two-dimensional gel electrophoresis (Penn, 1983). It was therefore possible to monitor the relative production of mN3 NS_2 mRNA and NS_1 mRNA in doubly infected cells indirectly by examining the pattern of virus-specific protein synthesis. Previous work had indicated that the pattern of protein synthesis in influenza virus-infected cells closely reflects that of mRNA accumulation (Hay *et al.*, 1977; Inglis *et al.*, 1979).

Cells were infected with both mN3 and ts47 or were infected separately with either mN3 or ts47, and were pulse-labelled with [^{35}S]methionine. A lysate of the mixedly infected cells (mixed infection) and a pooled lysate (separate infection) containing equal quantities of the cells separately infected with mN3 or ts47, were applied to two-dimensional polyacrylamide gels and radiolabelled proteins were detected by fluorography (Figure 3a and b). The relative amounts of NS_1 and NS_2 polypeptides were quantified by densitometer scanning (Table I). In both CEF and L cells, the synthesis of mN3 NS_2 relative to mN3 NS_1 was higher in the mixedly infected cells than in cells infected with mN3 alone, indicating that the production of mN3 NS_2 mRNA, relative to that of mN3 NS_1 mRNA, can be enhanced by products encoded by ts47. Since the ratio of mN3 NS_2mN3 NS_1 synthesis in mixedly infected cells was similar to that normally observed in wild-type virus-infected cells, it seems that the NS_1 mRNA encoded by mN3 does not contain any *cis*-acting mutations which affect its stability of suitability as a substrate for RNA splicing. These results support the idea that the mutant virus encodes a defective version of a product that normally regulates the production of NS_2 mRNA.

Effect of protein synthesis inhibitors on NS_2 mRNA production in wild-type and mutant virus-infected cells

To strengthen this conclusion further, we examined the production of NS_2 mRNA in cells that had been infected in the presence of the protein synthesis inhibitors cycloheximide and anisomycin. Under these conditions, NS_2 mRNA is produced inefficiently in wild-type virus-infected cells, suggesting that under normal circumstances, the production of NS_2 mRNA is promoted by virus-specific proteins (Lamb *et al.*, 1978; Inglis, 1978; Inglis and Brown, 1984). The effect of protein synthesis inhibitors on the relative production of NS_2 mRNA and NS_1 mRNA does not appear to result from a direct effect on RNA processing or stability, or to the reduced synthesis of a cellular protein, since NS_2 mRNA is produced normally in the presence of the inhibitors if the infected cells are first incubated for several hours in the absence of the drugs (Inglis and Brown, 1984). Hence, if mN3 does encode a defective version of a regulatory protein then it might be expected that the drugs would not reduce the production of NS_2 mRNA in mutant virus-infected cells.

Cytoplamsic extracts were prepared from untreated or drug-treated cells at 5 h (CEF) or 8 h (L cells) after infection, and the abundance of the vRNA segment 8-specific mRNAs in these extracts was monitored as before by hybrid selection of reverse transcripts (Figure 4). The results were quantified by densitometer scanning of the autoradiogram (Table II). Drug treatment of wild-type virus-infected CEF (lanes 1 and 2) or L cells (lanes 5 and 6) reduced the abundance of NS_2 mRNA relative to that of NS_1 mRNA, but in mN3-infected cells the relative abundance of NS_2 mRNA was either unaffected (CEF) (Lanes 3 and 4) or was increased (L cells) (lanes 7 and 8) by the drug treatment. These results suggest that the relative abundance of NS_2 mRNA is low in mN3-infected cells because the mutant encodes an altered version of a virus-specific product that is no longer able to regulate the production of NS_2 mRNA.

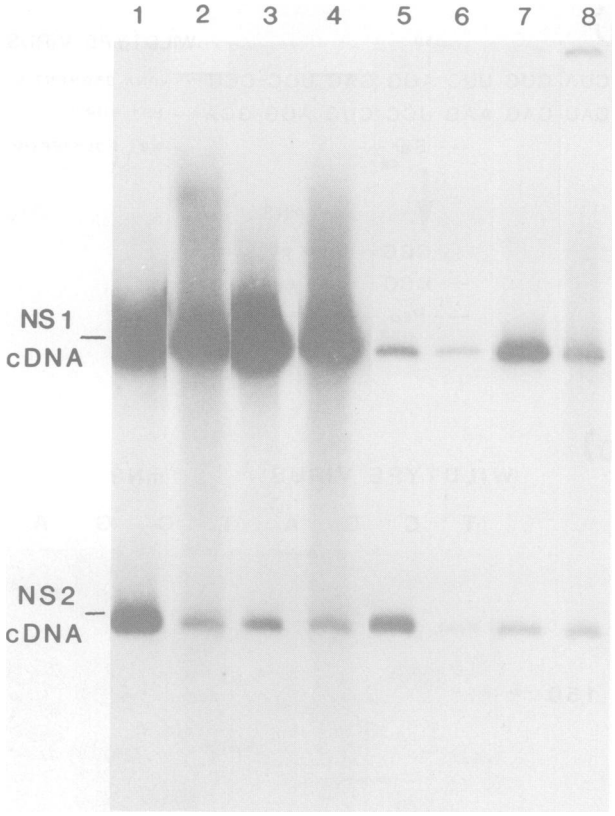

Fig. 4. Accumulation of vRNA segment 8-specific mRNAs in wild-type virus and mN3-infected cells in the absence of virus protein synthesis. CEF (**lanes 1–4**) and L cells (**lanes 5–8**) were infected with wild-type virus (**lanes 1, 2, 5** and **6**) or mN3 (**lanes 3, 4, 7** and **8**) at a multiplicity of 10 p.f.u./cell. Cells were either untreated (**lanes 1, 3, 5** and **7**) or were incubated in medium containing 100 μg/ml cycloheximide and 20 μg/ml anisomycin from 1 h before and throughout infection (**lanes 2, 4, 6** and **8**). Cytoplasmic extracts were prepared at 5 h (**lanes 1–4**) or 8 h (**lanes 5–8**) after infection and poly(A)-containing RNAs were reverse transcribed in the presence of [α-^{32}P]dCTP. Reverse transcripts of the vRNA segment 8-specific mRNAs were selected by hybridisation with nitrocellulose filters bearing DNA from the bacterial plasmid pFPV82A and detected by autoradiography after gel analysis. Autoradiography exposures are unequal and were chosen in order to facilitate the comparison of different samples.

Synthesis of NS_2 mRNA and NS_1 mRNA in wild-type and mutant virus-infected cells

In principle, there are several different means by which virus-specific products might regulate the production of NS_2 mRNA. One such possibility is that virus-specific products are required for the efficient transport of NS_2 mRNA from the nucleus to the cytoplasm; under conditions where the regulatory products were scarce, NS_2 mRNA would accumulate in the nucleus and so the

Table II. Proportion of segment 8-specified mRNA represented by NS_2 mRNA[a].

Infected cell type	Wild-type virus		mN3	
	no inhibitor	with inhibitor	no inhibitor	with inhibitor
CEF	17%	2.5%	2.3%	1.8%
L	67%	37%	12%	22%

[a]The density of the bands corresponding to NS_1 and NS_2 mRNA in Figure 4 was measured using a Beckman Du-8 spectrophotometer. The ratio NS_2/NS_2+NS_1 was then calculated.

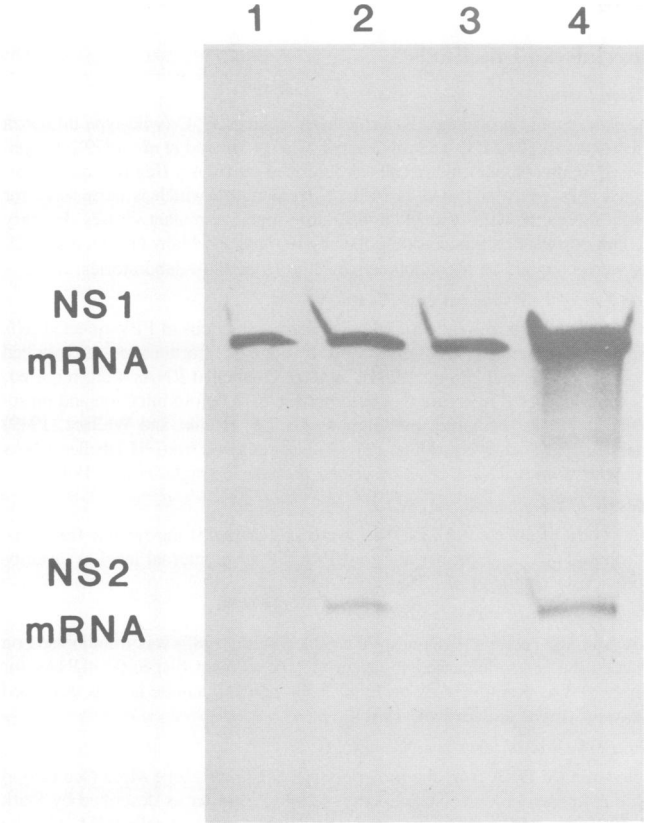

Fig. 5. Synthesis of vRNA segment 8-specific mRNAs in wild-type virus and mN3-infected CEF. Cells were infected with wild-type virus (**lanes 1** and **2**) or mN3 (**lanes 3** and **4**) at a multiplicity of 10 p.f.u./cell, and incubated in medium containing [^{3}H]uridine for 30 min from 3 h (**lanes 1** and **3**) or 5 h (**lanes 2** and **4**) after infection. Poly(A)-containing RNAs were isolated from the nuclear fraction of these cells and treated with RNAse H to remove poly(A). Equivalent amounts were hybridised with immobilised single-stranded DNA containing vRNA segment 8-specific sequences (m13 phage mp8 clone M3.3, Materials and methods), and specifically hybridising species were eluted and detected by fluorography after separation on a 7.5% polyacrylamide gel.

cytoplasmic abundance of NS_2 mRNA relative to that of NS_1 mRNA would be low. However, NS_2 mRNA did not accumulate preferentially in the nucleus of wild-type virus-infected CEF at early times after infection or at any time during infection with mN3 (data not shown).

Two potential means by which virus-specific products might regulate the production of NS_2 mRNA are by altering the relative stability of NS_2 mRNA and NS_1 mRNA, or by increasing the efficiency with which NS_1 mRNA serves as a substrate for RNA splicing. To try to distinguish between these possibilities, we examined the synthesis of NS_2 mRNA and NS_1 mRNA at different times after infection of CEF with either wild-type virus or mN3. If the production of NS_2 mRNA is regulated through changes in the relative stability of the vRNA segment 8-specific mRNAs, then the relative abundance of NS_2 mRNA and NS_1 mRNA amongst newly synthesised mRNAs might be expected to be similar at any time after infection.

Infected cells were pulse-labelled with [^{3}H]uridine for 30 min at either 3 or 5 h after infection and nuclear polyadenylated RNAs were isolated. Poly(A) was removed from these mRNAs by treatment with RNase H in the presence of oligo$(dT)_{12-18}$, and vRNA segment 8-specific RNAs were selected by hybridisation to immobilised single-stranded DNA from the bacteriophage M13 mp8 clone M3.3. This DNA contains sequences which correspond to those of wild-type virus vRNA segment 8 (Materials and methods) and so will only hybridise with virus RNAs of the positive sense. In wild-type virus-infected cells, synthesis of NS_2 mRNA relative to that of NS_1 mRNA increased between 3 and 5 h after infection, while in mN3-infected cells the relative synthesis of NS_2 mRNA was low even at 5 h after infection (Figure 5). These observations are consistent with the idea that the production of NS_2 mRNA is normally regulated through alterations in the efficiency with which NS_1 mRNA is processed by RNA splicing into NS_2 mRNA, and that this alteration does not occur in mN3-infected cells.

Discussion

The available evidence strongly favours the idea that cellular RNA processing enzymes are responsible for the splicing of vRNA segment 7- and 8-specified influenza virus mRNAs (Lamb and Lai, 1980, 1982, 1984; Lamb *et al.*, 1981). Previous work, however, has suggested that splicing of virus-specific mRNAs may be regulated during infection by virus-specific products. The basis for this proposal was the observation that spliced influenza virus mRNA, in particular the NS_2 mRNA, is produced inefficiently in the absence of virus-specific protein synthesis (Inglis and Brown, 1984; Lamb *et al.*, 1978); furthermore, the abundance of NS_2 mRNA relative to that of its unspliced precursor NS_1 mRNA, was found to increase during virus infection. The results presented here support the view that a virus-specified product (or products) regulates the production of NS_2 mRNA during infection, since in cells infected with the mutant virus mN3, amplification of NS_2 mRNA production does not occur. This deficiency in the regulation of NS_2 mRNA production is not simply the result of an alteration in the structure of its unspliced precursor leading to a failure in processing, since the sequences surrounding the splice sites in the mutant virus gene are unaltered, and since the RNA encoded by this mutant gene is spliced with normal efficiency during a mixed infection with a virus which is wild-type for NS_2 mRNA production. The results therefore suggest that a *trans*-acting factor, which is produced during virus infection as a result of virus-specified protein synthesis, acts to enhance the relative production of NS_2 mRNA late in infection.

It could be envisaged that this enhanced accumulation of NS_2 mRNA is due to an increase in the efficiency with which the NS_1 mRNA is spliced at late times after infection, or alternatively to a change in the turnover rate of the precursor or product RNA. Studies on the rates of synthesis of the two RNA species by pulse-labelling with [^{3}H]uridine suggested that the former possibility is more likely, since it was apparent that there was an increase in synthesis of the NS_2 mRNA, relative to that of NS_1 mRNA, during the time course of infection. However, our results cannot exclude entirely the possiblity that NS_2 mRNA production

is regulated through changes in the relative stability of the vRNA segment 8-specific mRNAs, since the effects of such changes might become manifest within the 30-min labelling period employed for the experiment. For example, the relative abundance of NS_2 mRNA and NS_1 mRNA might change during infection in wild-type virus-infected cells, because of an increase in the degradation of NS_1 mRNA and this increase might fail to occur with mutant virus. Indeed it was apparent that in mutant-infected CEF, the overall synthesis and also accumulation of vRNA segment 8-specific mRNA was higher than with wild-type virus. However, this explanation is not consistent with our finding that in mutant-infected L cells, where NS_2 mRNA production was clearly deficient, there was no enhanced accumulation of NS_1 mRNA. The higher rate of vRNA segment 8 transcription observed in mutant-infected CEF may therefore be unrelated to the deficiency in regulation of NS_2 mRNA production. The difficulty of distinguishing between changes in RNA stability and changes in processing efficiency also applies to other studies of the regulation of spliced mRNA production (Nevins and Wilson, 1981; Inglis and Brown, 1984) although in one case there is evidence that regulation is mediated through changes in RNA stability (Wilson and Darnell, 1981).

If it is indeed the case that virus-specific products promote the splicing of NS_1 mRNA late in infection, we can imagine a number of ways in which this might occur. A virus protein (or RNA) might interact with cell splicing enzymes late in infection, directly affecting the efficiency with which the RNA is spliced. Another, more plausible, possibility is that enhanced splicing of virus mRNA reflects a change in the way in which the precursor RNA is presented as a substrate for cellular processing enzymes. Changes in the sites of synthesis of virus-specific mRNA, or in its interaction with protein, could alter the proportion which is spliced, as could changes in the nuclear architecture following virus infection. Indeed previous work suggests that the particular cellular context in which virus-specific transcripts are produced may affect the efficiency with which they are processed; a much higher proportion of vRNA segment 7- and 8-specific transcripts are spliced in FPV-infected mouse L cells than in CEF cell (Inglis and Brown, 1984), and in cells infected with a recombinant SV40 virus containing a cloned copy of influenza vRNA segment 8, ~50% of the influenza-specific transcripts detected in the cells were spliced (Lamb and Lai, 1984) while in L cells containing stable integrated copies of the same gene, >90% of the transcripts were found to be spliced (M. Inglis, personal communication).

While our results indicate that virus factors act to promote the production of NS_2 mRNA, they also suggest that this amplification is not necessary for virus growth, at least in tissue culture. The mutant virus mN3 is temperature-sensitive, but its failure to amplify production of NS_2 mRNA, is apparent at both restrictive and permissive temperatures. A temperature-sensitive lesion has been mapped previously to vRNA segment 8 of mN3 (Almond *et al.*, 1979) and so presumably resides in the mutation at nucleotide 150 which results in the substitution of a proline for a serine at residue 42 of the mN3 NS_1 polypeptide. However, we cannot with certainty ascribe the defect in the regulation of NS_2 mRNA production to this mutation, since there may be other changes in the mutant virus genome. We have attempted to investigate this point further through the analysis of reassortant viruses between two strains of FPV which differ in production of NS_2 mRNA. The results indicated that the efficiency of NS_2 mRNA production generally segregates with vRNA segment 8, but there were exceptions to this pattern and so we cannot be certain that vRNA segment 8 encodes a regulatory product. One possibility is that the production of NS_2 mRNA is regulated through the concerted action of several virus-specific products, one of which may be the NS_1 protein.

These same virus-specific products may also regulate the production of the vRNA segment 7-specific spliced mRNAs (M_2 and M_3 mRNA) since there is evidence that virus-specific products are required for the efficient production of these mRNAs in infected CEF (Inglis and Brown, 1984). However, the production of M_2 mRNA and M_3 mRNA is not obviously deficient in mN3-infected CEF (unpublished results) and it is possible that the production of these spliced mRNAs is regulated differently.

Materials and methods

Cells and virus

Virus stocks were propagated in fertile hens' eggs at 37°C (wild-type influenza A/FPV/Rostock) or 34°C (tsC mN3 and TsC 47) (Almond *et al.*, 1979), assayed and used to infect tissue culture cells as described previously (Inglis *et al.*, 1976). Infected cells were incubated at 34°C, a temperature which is permissive for growth of both the wild-type and temperature-sensitive mutant viruses. Primary CEF cultures were prepared as described by Borland and Mahy (1968), and L929 cells were obtained as a continuous cell line from Flow laboratories.

Extraction of FPV-infected cell RNA

RNA was extracted from nuclear and cytoplasmic fractions of FPV-infected cells and poly(A)-containing RNAs were isolated by oligo(dT) fractionation, as described previously (Inglis and Brown, 1984). Where ^{3}H-labelled RNAs were required, cells were incubated in serum-free medium for 12 h before infection and pulse-labelled in Hanks' balanced salt solution (pH 7.6) (Hanks and Wallace, 1949) containing 50 μCi/ml [^{3}H]uridine. Poly(A) was removed from ^{3}H-labelled RNAs by treatment with RNase H as described previously (Inglis *et al.*, 1980).

Synthesis of complementary DNA

DNA copies of infected cell mRNAs were synthesised using reverse transcriptase, oligo$(dT)_{12-18}$ as primer and [α-^{32}P]dCTP as an internal label as described previously (Inglis *et al.*, 1980).

Hybrid selection of virus-specific cDNAs

DNA from the bacterial plasmid pFPV 82A (Smith, 1985) was immobilised on nitrocellulose filters and used to select vRNA segment 8-specific cDNAs for analysis of Tris-borate-EDTA-buffered 7.5% polyacrylamide gels as described previously (Inglis and Brown, 1981).

Hybrid selection of virus-specific mRNAs

Single-stranded DNA from the bacteriophage M13 mp8 clone M3.3 (see below) was immobilised on diazobenzylmethyl cellulose powder as described by Stark and Williams (1979). Hybridisation of ^{3}H-labelled infected cells RNA with the powder and elution of specifically hybridising species was as described by Smith *et al.* (1979). Gel analysis was as described in Inglis and Brown (1981) except that samples were heated at 95°C for 30 s before loading. Labelled species were detected by fluorography.

Analysis of infected cells protein synthesis

Monolayers of infected cells were rinsed with PBS and pulse-labelled with [^{35}S]methionine (200 μCi/ml) in methionine-free medium. Cells were dislodged into lysis buffer (9.5 M urea, 5% β-mercaptoethanol, 2% LKB ampholines pH 3.5–10, adjusted to pH 4.0 with H_3PO_4) and subjected to centrifugation at 100 000 *g* for 1 h at 10°C in order to pellet DNA. Samples were analysed by two-dimensional non-equilibrium pH gradient electrophoresis as described by O'Farrell *et al.* (1977), except that the first dimension gel contained 5% LKB ampholines pH 3.5–10 and 2% LKB ampholines pH 5–7, and electrophoresis was for 5 h at 500 V. Separation in the second dimension was through a 17.5% SDS-polyacrylamide gel at 50 V for 12 h.

Molecular cloning of vRNA segment 8

Virion RNA from mN3 and wild-type virus was purified and copied into double-stranded DNA as described by Roditi and Robertson (1984) in reactions catalysed by reverse transcriptase and *Escherichia coli* DNA polymerase I and primed by oligonucleotides complementary to the 3′ terminus of the vRNA segments or to the 3′ terminus of their cDNA copies. Double-stranded DNA copies of vRNA segment 8 were isolated after electrophoresis through a 1% low gelling temperature agarose gel and covalently joined with replicative form DNA of bacteriophage M13 mp8 at the *Sma*I restriction site as described by Bankier and Barrell (1983). Transfection of *E. coli* strain TG1 (Gibson, 1984) was as described

by Hanahan (1983). Recombinant bacteriophage containing vRNA segment 8-specific sequences were detected by hybridisation of ^{32}P-labelled DNA from the bacterial plasmid pISI 86 (Roditi, 1983) with nitrocellulose filters bearing immobilised bacteriophage DNA. The conditions for hybridisation were as described by Inglis and Brown (1981).

Nucleotide sequencing

The nucleotide sequence of vRNA segment 8-specific regions in the recombinant M13 mp8 bacteriophage was deduced by the dideoxynucleotide chain termination procedure (Sanger *et al.*, 1977) as described by Bankier and Barrell (1983). Sequencing reactions employed [α-^{32}P]dCTP as the radioactive label and were analysed after electrophoresis on buffer gradient polyacrylamide gels (Biggin *et al.*, 1983). The nucleotide sequences of wild-type virus and mN3 vRNA segment 8 were deduced from (i) the partial sequence of cloned copies of vRNA segment 8 and (ii) the sequence of subcloned fragments of a single wild-type virus and a single mN3-derived clone. These subclones were produced by isolating *Taq*I restriction fragments of the cloned copy of vRNA segment 8 and joining these fragments with M13 mp8 replicative form DNA at the *Acc*I restriction site. Most regions were sequenced on only one strand and no attempt was made to cross the *Taq*I restriction sites since the complete nucleotide sequence of FPV vRNA segment 8 had been deduced previously (Porter *et al.*, 1980).

The reaction conditions for direct sequencing of vRNA segment 8 by the dideoxynucleotide chain termination procedure were as described by Rogers *et al.* (1983) except that virion RNA was first fractionated on a 4% polyacrylamide gel and vRNA segment 8 was recovered from the gel by soaking in 0.5 M ammonium acetate, 0.5% SDS, 1 mM EDTA for 12 h at 37°C. Reverse transcription was primed with the oligonucleotide (dAGCAAAAGCAGG) which is complementary to the vRNA 3′ terminus and reaction products were separated on a 6% polyacrylamide buffer-gradient gel (Biggin *et al.*, 1983).

One of the recombinant M13 mp8 bacteriophage clones (M3.3) contained sequences derived from, and in the same sense as, wild-type virus vRNA segment 8. This clone was used for the hybrid selection of ^{3}H-labelled vRNA 8-specific mRNAs as described above.

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